

# RAGE Mediates a Novel Proinflammatory Axis: A Central Cell Surface Receptor for S100/Calgranulin Polypeptides

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## Summary

S100/calgranulin polypeptides are present at sites of inflammation, likely released by inflammatory cells targeted to such loci by a range of environmental cues. We report here that receptor for AGE (RAGE) is a central cell surface receptor for EN-RAGE (extracellular newly identified RAGE-binding protein) and related members of the S100/calgranulin superfamily. Interaction of EN-RAGEs with cellular RAGE on endothelium, mononuclear phagocytes, and lymphocytes triggers cellular activation, with generation of key proinflammatory mediators. Blockade of EN-RAGE/RAGE quenches delayed-type hypersensitivity and inflammatory colitis in murine models by arresting activation of central signaling pathways and expression of inflammatory gene mediators. These data highlight a novel paradigm in inflammation and identify roles for EN-RAGEs and RAGE in chronic cellular activation and tissue injury.

## Introduction

The receptor for advanced glycation end products (RAGE) is a multiligand member of the immunoglobulin superfamily of cell surface molecules (Neeper et al., 1992; Schmidt et al., 1992). RAGE was originally identified and characterized based on its ability to bind advanced glycation end products (AGEs), adducts formed by glycoxidation that accumulate in disorders such as diabetes and renal failure (Brownlee et al., 1988; Miyata et al., 1996). The proximity of cells expressing RAGE to lesional areas rich in AGEs, and the ensuing cellular activation, suggested the possibility that AGE–RAGE in-

teraction might trigger intracellular signal transduction mechanisms altering properties of vascular and inflammatory effector cells, thereby contributing to impaired reparative responses in AGE-rich tissues, as occurs in diabetes (King and Brownlee, 1996). Indeed, AGE ligation of RAGE activates p21<sup>ras</sup>, recruiting downstream targets, such as MEK and MAP kinases, and activating the transcription factor NF- $\kappa$ B; this represents the first receptor-dependent signal transduction pathway for AGEs (Lander et al., 1997). Subsequent studies demonstrating that RAGE could serve as a cell surface receptor for amyloid- $\beta$  peptide (A $\beta$ ) (Yan et al., 1996, 1997), a cleavage product of the  $\beta$ -amyloid precursor protein that accumulates and has been ascribed a pathogenic role in Alzheimer's disease (Selkoe, 1994), extends the concept of RAGE as a receptor that converts proteinaceous deposits, of glycoxidized adducts or A $\beta$ , into bioactive species capable of modulating cellular properties. This view of the biology of RAGE contrasts with the effective uptake and disposal of AGEs, A $\beta$ , and other ligands by the scavenger receptor (Krieger and Herz, 1994); RAGE is much less efficient in mediating endocytosis and degradation of bound ligands (Mackic et al., 1998), but, rather, ligand–receptor interaction causes cellular perturbation (Wautier et al., 1996; Park et al., 1998).

Expression of RAGE at high levels during central nervous system development and the observation that lung was a rich source of RAGE in adult animals suggested quite different roles for the receptor than might be expected if the molecule evolved solely to interact with AGEs and A $\beta$ . In fact, a nonglycated polypeptide, amphoterin, is a RAGE ligand present in developing brain in an overlapping distribution with cells expressing the receptor (Hori et al., 1995). In contrast to this physiologic expression and, possibly, function of RAGE in development, the presence of receptor in the lung raised the question of a contribution of RAGE in the response to environmental challenge. Our analysis of lung tissue for "natural" ligands of RAGE has yielded an intriguing answer to this question and has provided insight into a novel axis in the biology of inflammation; RAGE is a signal transduction receptor for S100/calgranulin-like molecules.

The S100/calgranulin family is comprised of closely related polypeptides released from activated inflammatory cells, including polymorphonuclear leukocytes, peripheral blood-derived mononuclear phagocytes, and lymphocytes (Zimmer et al., 1995; Schafer and Heinzmann, 1996). Their hallmark is accumulation at sites of chronic inflammation. To date, however, specific means by which these polypeptides modulate the course of inflammatory processes have not been elucidated. We report here the characterization of an ~12 kDa polypeptide, termed EN-RAGE (extracellular newly identified RAGE-binding protein), which is in the S100/calgranulin family. Ligation of cellular RAGE by EN-RAGE and EN-RAGE-like molecules (another S100 family member) mediates activation of endothelial cells, macrophages, and lymphocytes, cells central to the inflammatory response.

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Exhibit B

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Table 1. Amino Acid Sequence Analysis of p12, Later Termed "EN-RAGE," and Comparisons with Homologous Polypeptides

	1	10	20	30
P12 N TERM	T K L E D H L E G I I N I F H Q Y S V R V G H F D T L N K Y			
P12 CNBR	T K L E D H L E G I I N I F H Q Y S V R V G H F D T L N K R			
B-COAg	T K L E D H L E G I I N I F H Q Y S V R V G H F D T L N K R			
B-CAAF1	T K L E D H L E G I I N I F H Q Y S V R V G H F D T L N K R			
	31	40	50	60
P12 N TERM	E L K Q L G T K E L P K T L Q N X K D Q			
P12 CNBR	E L K Q L I T K E L P K T L Q N T K D Q P T I D K I F Q D L			
B-COAg	E L K Q L I T K E L P K T L Q N T K D Q P T I D K I F Q D L			
B-CAAF1	E L K Q L I T K E L P K T L Q N T K D Q P T I D K I F Q D L			
	61	70	80	90
P12 N TERM		D G A V S F E E F V V L V S R V L K		
P12 CNBR		D A D K D G A V S F E E F V V L V S R V L K T A H I D I H K		
B-COAg		D A D K D G A V S F E E F V V L V S R V L K T A H I D I H K		
B-CAAF1		D A D K D G A V S F E E F V V L V S R V L K T A H I D I H K		

The amino acid sequence analysis of EN-RAGE was compared with homologous polypeptides bovine corneal antigen and bovine CAAF1. The latter sequences were obtained from Gottsch et al. (1997). A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. X is an amino acid residue not identified at that position.

Consistent with the concept that EN-RAGE interaction with RAGE contributes importantly to a proximal step in the cascade of events amplifying inflammation, blockade of RAGE suppressed inflammation in acute and chronic models. In parallel, inhibition of RAGE-S100/calgranulin interaction decreased NF- $\kappa$ B activation and expression of proinflammatory cytokines in tissues, suggesting that receptor blockade altered the course of the inflammatory response. These data thus highlight a novel paradigm in inflammation and identify roles for EN-RAGEs and RAGE in chronic cellular activation and tissue injury.

## Results

### Characterization of the ~12 kDa RAGE-Binding Protein

The evolution of an immunoglobulin superfamily molecule solely to engage the products of nonenzymatic glycoxidation or A $\beta$  seemed improbable, especially in view of the multiligand specificity of certain other members of this family (Springer, 1990). We thus considered the possibility that RAGE would recognize other physiologically/pathophysiologically relevant ligands. As RAGE was expressed at highest levels in the lung, this tissue seemed a logical place to launch a search for natural ligands. Indeed, bovine lung extract displayed binding activity for RAGE, which, following column chromatography, was resolved into two discrete polypeptides. The first was identified as amphoterin, a high mobility group 1 nonhistone nuclear protein also present extracellularly (Rauvala and Pihlaskari, 1987). Amphoterin participates in cell matrix interactions, mediated at least in part by RAGE, and, because of its high levels in the embryonic period, amphoterin-RAGE interaction might prove important for guiding developmental processes (Hori et al., 1995). The second RAGE-binding species, an ~12 kDa polypeptide, was subjected to N-terminal and internal sequence analysis, the latter following digestion with the endopeptidase Lys-C (Table 1). When first compared with sequences available in the database in 1995, the sequence obtained was unique, leading us to assign it

the name EN-RAGE. Subsequently, sequences entered in the database showed EN-RAGE to bear striking homology to a polypeptide known as bovine calcium-binding protein in amniotic fluid-1 (CAAF-1; Hitomi et al., 1996) or bovine corneal antigen/calgranulin C (Gottsch et al., 1997), recently classified as S100A12 (Ilg et al., 1996). Although the protein sequence of EN-RAGE differed at two positions (amino acids 30 and 36) from that of bovine corneal antigen (Table 1), molecular cloning studies firmly placed EN-RAGE in the calgranulin/S100 family (Dell'Angelica et al., 1994; Ilg et al., 1996; Wicki et al., 1996; Gottsch et al., 1997) and led to isolation of clones identical to the deduced amino acid sequence of bovine corneal antigen. Analysis of the sequence indicated that EN-RAGE was most likely the bovine counterpart of human calgranulin C (or human corneal antigen/S100A12) (Ilg et al., 1996; Yamamura et al., 1996), displaying >77% homology. Since the term EN-RAGE described a potentially central property of the ~12 kDa polypeptide, and, potentially other S100/calgranulins, their interaction with RAGE, we have retained this name at this time, at least until nomenclature for members of this family is standardized. We thus exploited implications of the term "EN-RAGE" to determine whether the EN-RAGE/RAGE axis provides a critical, previously unrecognized link between the family of S100/calgranulin proteins and a signal transduction receptor permitting them to exert cellular effects.

### EN-RAGE Expressed by Inflammatory Cells Stimulates Cellular Activation via RAGE

Members of the S100/calgranulin family have been associated with a range of inflammatory disorders, especially those of a chronic nature. These polypeptides are present at inflammatory loci, most likely due to their release by inflammatory effector cells, such as polymorphonuclear leukocytes and peripheral blood-derived mononuclear phagocytes. First, it was important to demonstrate upregulation of EN-RAGE in stimulated inflammatory effector cells and then to show their release following appropriate stimulation. Peripheral blood-derived mononuclear cells (PBMCs) or Jurkat cells, an immortalized

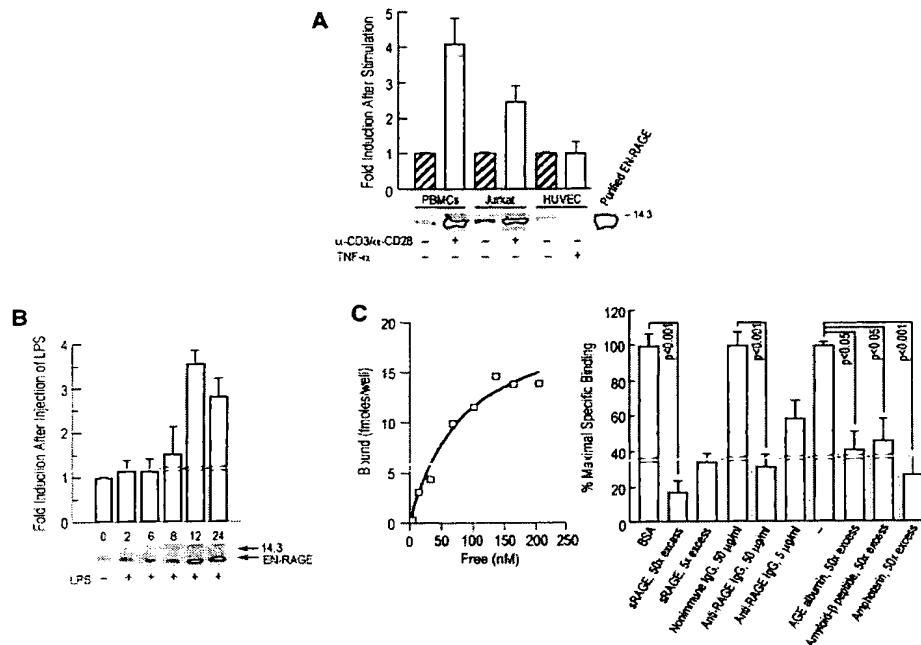


Figure 1. Expression of EN-RAGE Is Increased in Stimulated Inflammatory Cells, and EN-RAGE Binds RAGE

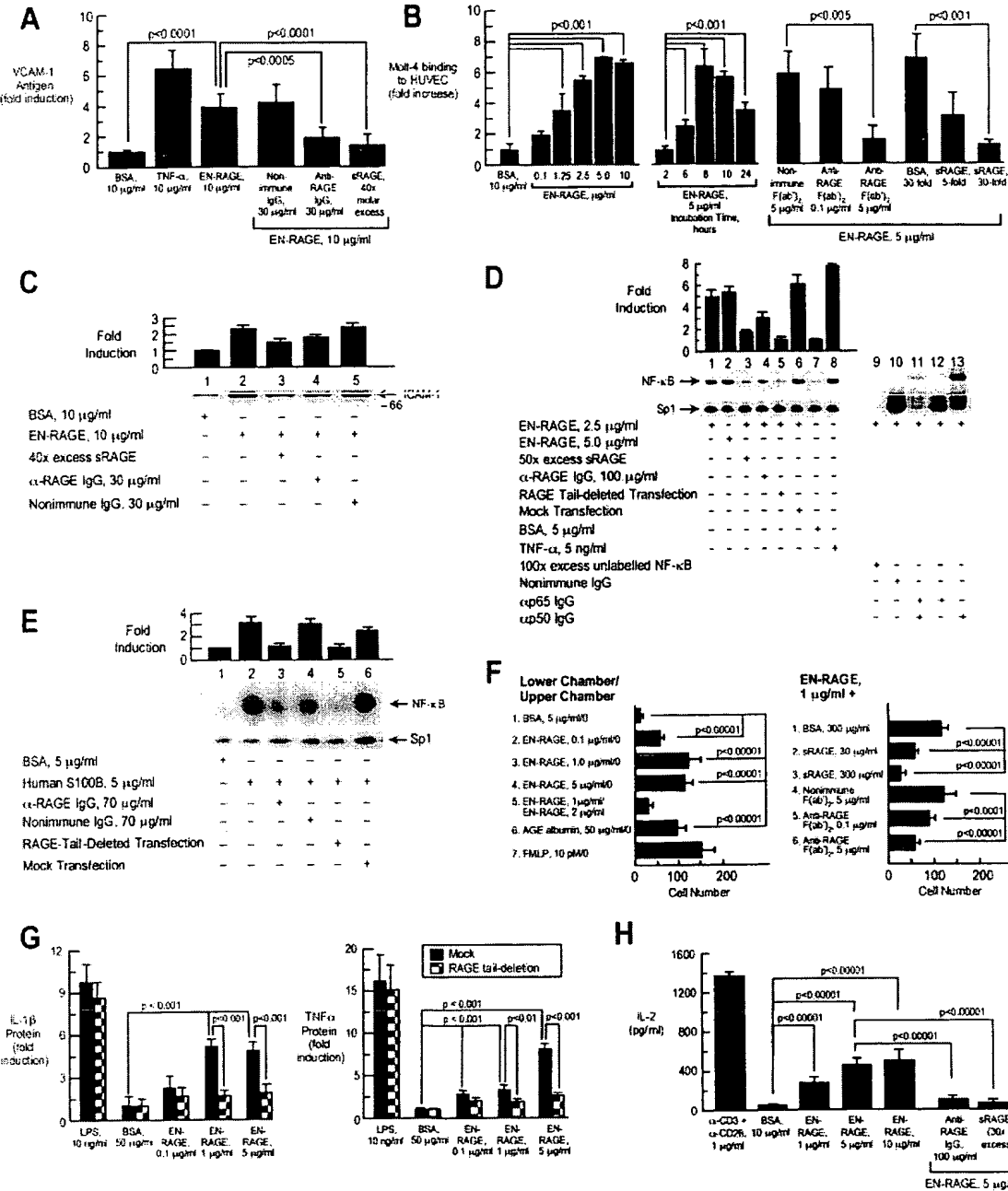
(A) Expression of EN-RAGE in stimulated PBMCs and Jurkat cells. PBMCs, Jurkat E6 cells, or HUVECs were cultured alone or in the presence of the indicated stimuli. Cell lysates were prepared and electrophoresis/immunoblotting performed employing rabbit anti-EN-RAGE IgG (2  $\mu$ g/ml). Levels of IL-2 elaborated into cellular supernatant were determined by ELISA to control for extent of stimulation by cross-linking CD3/CD28. In PBMCs (-/+ stimulation), levels of IL-2 were  $30 \pm 7$  and  $750 \pm 35$  pg/ml, respectively. In Jurkat cells (-/+ stimulation), levels of IL-2 were  $40 \pm 7$  and  $2100 \pm 70$  pg/ml, respectively. (B) Infusion of LPS into mice results in elaboration of EN-RAGE into plasma. LPS (30  $\mu$ g/kg body weight) was infused into CF-1 mice. At the indicated time, blood was retrieved and plasma was subjected to electrophoresis/immunoblotting for EN-RAGE using anti-EN-RAGE IgG as above. In (A) and (B), results of densitometric analysis and mean  $\pm$  SD of three experiments are shown. (C) EN-RAGE binds purified RAGE. Murine soluble RAGE was immobilized onto the wells of plastic dishes. Radioligand binding assays were performed employing the indicated concentration of  $^{125}$ I-EN-RAGE in the presence or absence of excess unlabeled EN-RAGE (50-fold). Specific binding to purified RAGE is demonstrated, with  $K_D = 91 \pm 29$  nM and capacity  $\approx 21 \pm 2.9$  fmol/well. Where indicated, radiolabeled EN-RAGE (100 nM) was preincubated with either sRAGE, or wells were incubated with alternate RAGE ligand (50-fold molar excess in each case). Alternatively, wells were preincubated with the indicated concentration of nonimmune IgG, or anti-RAGE IgG prior to binding assay. Results are reported as percent of maximal specific binding  $\pm$  SD.

T cell line, stimulated by cross-linking CD3/CD28, displayed increased EN-RAGE in cellular homogenates, corresponding to  $\sim 4.1$ -fold and 2.5-fold, respectively (Figure 1A). In contrast, stimulation of cultured human umbilical vein endothelial cells (HUVECs) with a prototypic stimulus, tumor necrosis factor  $\alpha$  (Figure 1A), did not modulate expression of EN-RAGE. To address the second issue—the extracellular release of EN-RAGE—mice were infused with lipopolysaccharide (LPS), and plasma was assayed for EN-RAGE. Time-dependent release of EN-RAGE into plasma was noted with maximal levels by 12 hr of injection, corresponding to an  $\sim 3.6$ -fold increase (Figure 1B).

To directly test the concept that EN-RAGE bound RAGE, radioligand binding studies were performed. Specific binding of  $^{125}$ I-EN-RAGE to purified RAGE on plastic wells was dose dependent, with  $K_D \approx 91 \pm 29$  nM (Figure 1C). Specificity of binding was shown by inhibition in the presence of excess soluble RAGE (sRAGE), a truncated form of the receptor spanning the extracellular domain, or anti-RAGE IgG. In contrast, unrelated proteins, bovine serum albumin, or nonimmune

IgG were without effect. Further, alternate ligands for RAGE, amphotericin, AGE albumin, and amyloid- $\beta$  peptide, similarly inhibited binding of EN-RAGE to immobilized RAGE (Figure 1C, inset). The interaction of S100/calgranulin polypeptides with RAGE was not limited to EN-RAGE, as S100B also suppressed  $^{125}$ I-EN-RAGE interaction with RAGE (data not shown). Similar radioligand binding experiments were performed with RAGE in its natural cellular environment using cultured endothelial cells that endogenously express cell surface RAGE. Cultured endothelial cells displayed saturable binding of  $^{125}$ I-EN-RAGE, with  $K_D = 90.3 \pm 34$  nM; binding was suppressed in the presence of either excess sRAGE or anti-RAGE IgG (data not shown).

Upregulation of EN-RAGE, its release in response to inflammatory stimuli, and its ability to bind RAGE-bearing cells important in the inflammatory response suggested the possible relevance of EN-RAGE-RAGE interaction in the pathogenesis of inflammatory lesions. We explored the effect of EN-RAGE on properties of RAGE-bearing cells in order to determine whether RAGE-



**Figure 2.** Ligand of RAGE by EN-RAGE and S100B Results in Activation of ECs (A-E), MPs (F and G), and PBMCs (H)

(A) Assessment of VCAM-1. HUVECs were incubated with the indicated mediators for 8 hr in the presence or absence of pretreatment with nonimmune IgG, anti-RAGE IgG, or excess soluble RAGE. Cells were fixed, and cell surface ELISA for VCAM-1 was performed.

(B) Molt-4 adhesion assays. HUVECs were incubated with the indicated mediators for 8 hr. Varying concentrations (left panel) and incubation times (middle panel) for EN-RAGE were employed. After incubation, <sup>51</sup>chromium-labeled Molt-4 cells were bound to the monolayer for 1 hr, and cells were then disrupted in the presence of Triton X-100 (1%); the resulting material was counted in a beta counter. In the right panel, HUVECs were treated with EN-RAGE in the presence or absence of pretreatment with the indicated F(ab')<sub>2</sub>, excess sRAGE, or excess BSA. Results are reported as fold increase above control (treatment with BSA). In (A) and (B), results are reported as mean ± SD.

(C) Assessment of ICAM-1. HUVECs were incubated with the indicated mediators in the presence or absence of pretreatment with nonimmune IgG, anti-RAGE IgG, or excess soluble RAGE. Cell extract was obtained, and immunoblotting for ICAM-1 was performed. Mean ± SD of three experiments is shown.

dependent responses underlying inflammation might occur.

#### Endothelial Cells

Endothelium (HUVECs) incubated with EN-RAGE displayed induction of vascular cell adhesion molecule-1 (VCAM-1) (Figure 2A), a cell adhesion molecule that tethers mononuclear cells bearing VLA-4 to the vessel surface (Li et al., 1993). The key role of RAGE in EN-RAGE-mediated induction of endothelial VCAM-1 was shown by inhibition in the presence of anti-RAGE IgG or sRAGE (Figure 2A). The functional significance of VCAM-1 expression by EN-RAGE-treated endothelium was shown by enhanced binding of VLA-4-bearing Molt-4 cells (Figure 2B). Adherence of Molt-4 cells to EN-RAGE-treated endothelium was dependent both on the concentration and incubation time of EN-RAGE (left and middle panels, respectively) and required interaction of EN-RAGE with the receptor, as shown by the inhibitory effect of anti-RAGE F(ab')<sub>2</sub> and sRAGE (right panel). In addition to mechanisms by which mononuclear cells might be targeted to endothelium upon ligation of RAGE by EN-RAGE, enhanced expression of intercellular adhesion molecule-1 (ICAM-1) upon incubation of HUVECs with EN-RAGE was noted in a RAGE-dependent manner (Figure 2C), thereby providing a mechanism by which polymorphonuclear leukocytes might too be attracted to EN-RAGE-stimulated endothelium. Expression of VCAM-1 and ICAM-1 in response to inflammatory stimuli is subject, at least in part, to regulation at the transcriptional level by nuclear factor- $\kappa$ B (NF- $\kappa$ B) (Voraberger et al., 1991; Neish et al., 1992). We previously demonstrated that ligation of RAGE by AGEs and amyloid- $\beta$  peptide enhanced nuclear translocation of NF- $\kappa$ B, as demonstrated by electrophoretic mobility shift assay (EMSA) (Yan et al., 1994, 1996; Lander et al., 1997). Nuclear extracts from endothelium exposed to EN-RAGE were subjected to EMSA with a <sup>32</sup>P-labeled NF- $\kappa$ B probe and showed NF- $\kappa$ B activation (Figure 2D). Over the same concentration range that induction of VCAM-1 expression and enhanced Molt-4 cell binding was observed, endothelial cells exposed to EN-RAGE displayed an ~5-fold increase in the intensity of the gel shift band compared with cultures incubated with albumin (Figure 2D, lanes 1, 2, and 7, respectively; note densitometric analysis of bands upon normalization for Sp1). That this was largely mediated by EN-RAGE interaction with RAGE was confirmed by the inhibitory effect of anti-RAGE IgG or sRAGE added to the endothelium prior

to EN-RAGE (Figure 2D, lanes 4 and 3, respectively). Supershift assays with anti-p50 and anti-p65 IgG demonstrated that the NF- $\kappa$ B complex activated upon ligation of RAGE by EN-RAGE was composed of both p50 and p65 (Figure 2D, lanes 11–13). EN-RAGE induction of NF- $\kappa$ B nuclear translocation resulted from RAGE-mediated intracellular signaling, as shown by experiments using a truncated form of the receptor, termed dominant-negative RAGE (DN-RAGE), from which the cytosolic tail was deleted. Endothelium transfected with DN-RAGE displayed marked suppression of NF- $\kappa$ B activation (Figure 2D, lane 5) compared with those transfected with vector alone (Figure 2D, lane 6). Together, these data suggested that EN-RAGE interaction with endothelial RAGE activated NF- $\kappa$ B, potentially triggering expression of multiple gene products contributing to the inflammatory response, such as VCAM-1 and ICAM-1.

In view of the strong homology between members of the S100/calgranulin family, we sought to determine whether another family member, human S100B, would mediate RAGE-dependent endothelial activation of NF- $\kappa$ B. Using EMSA with <sup>32</sup>P-labeled NF- $\kappa$ B probe, nuclear extracts from HUVECs incubated with S100B showed an ~3.2-fold increased intensity of the gel shift band (Figure 2E, lane 2) compared with cultures exposed to BSA (Figure 2E, lane 1). That these findings were due to activation of RAGE was demonstrated by the inhibitory effect of anti-RAGE IgG (Figure 2E, lane 3), overexpression of DN-RAGE (Figure 2E, lane 5), and excess sRAGE (data not shown). Control experiments in which endothelial cultures were preincubated with nonimmune IgG (Figure 2E, lane 4) or mock transfection was performed (Figure 2E, lane 6) showed no effect on S100B-RAGE-induced NF- $\kappa$ B activation. These observations emphasize the likelihood that a range of S100/calgranulin polypeptide ligands engage RAGE.

#### Mononuclear Phagocytes

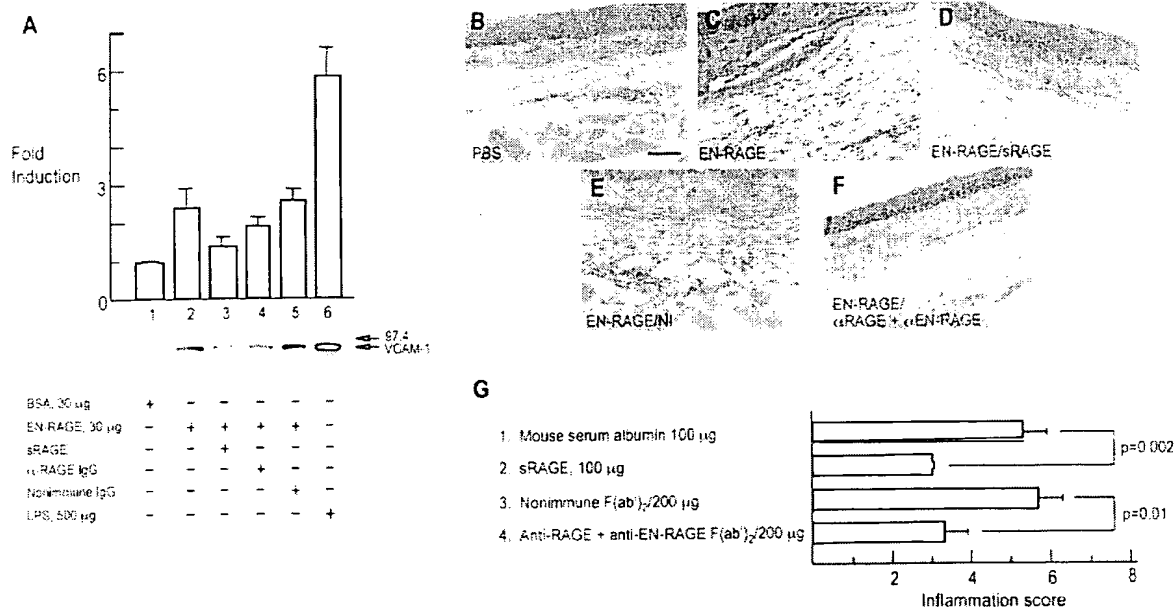
Release of EN-RAGE at sites of an ongoing inflammatory response could serve both to propagate and amplify the cellular response by recruiting mononuclear phagocytes (MPs). In this regard, two issues were critical: EN-RAGE-mediated induction of MP migration, and activation, following ligation of RAGE. Using modified chemotaxis chambers, EN-RAGE placed in the lower compartment stimulated migration of RAGE-bearing peripheral blood-derived human monocytes added to the upper compartment in a dose-dependent manner (Figure 2F, left panel, lines 2–4). In contrast, replacement of EN-RAGE with

(D and E) Electrophoretic mobility shift assay. HUVECs were treated with the indicated mediators for 8 hr. EN-RAGE (D) or S100B-treated (E) cells were preincubated with anti-RAGE IgG; EN-RAGE was pretreated with excess sRAGE. Certain HUVECs were transiently transfected with a construct encoding DN-RAGE or with vector control prior to treatment with EN-RAGE or S100B. Nuclear extract was prepared and EMSA performed employing radiolabeled probes for NF- $\kappa$ B and Sp1. Supershift assays were performed by incubation of nuclear extract with the indicated antibody prior to EMSA. Results of densitometric analysis after normalization for Sp1 are indicated; mean  $\pm$  SD of three experiments is shown.

(F) Modified chemotaxis assays. Mediators were placed in the upper or lower chamber, and human peripheral blood-derived monocytes were placed in the upper chamber for 4 hr. Cells that had migrated through the membranes were stained and counted. Where indicated (right panel), cells were pretreated with the indicated F(ab')<sub>2</sub> fragments or EN-RAGE incubated with excess sRAGE prior to chemotaxis assay. Mean  $\pm$  SD is shown.

(G) Generation of IL-1 $\beta$  and TNF $\alpha$ . BV-2 macrophages, either those transfected with DN-RAGE or mock-transfected cells, were incubated with the indicated mediators for 8 hr. Supernatant was collected, and ELISA for IL-1 $\beta$  or TNF $\alpha$  was performed. Mean  $\pm$  SD is shown.

(H) Generation of IL-2. PBMCs were incubated with the indicated mediators for 8 hr; supernatant was collected, and ELISA for IL-2 was performed. Where indicated, cells were pretreated with the indicated IgG, or EN-RAGE was pretreated with excess sRAGE. Mean  $\pm$  SD is reported. In (G), results are reported as fold induction (incubation of cells with BSA alone).



**Figure 3. EN-RAGE Mediates Cellular Activation and Inflammation In Vivo**

(A) Expression of VCAM-1 in the lung. CF-1 mice were injected with EN-RAGE, BSA, or LPS in the presence or absence of RAGE blockade. Twelve hours later, lungs were harvested, and immunoblotting for VCAM-1 was performed. Mean densitometric analysis  $\pm$  SD of three experiments is shown. (B–G) Injection of EN-RAGE into mouse footpad induces inflammation. (B–F) H&E analysis. Unilateral hind footpad was injected with the indicated mediator; mice were treated with either sRAGE, MSA, or anti-RAGE/anti-EN-RAGE F(ab)<sub>2</sub> and representative H&E-stained sections shown. Bar, 75 µm. (G) Inflammation score. Twenty-four hours after injection, clinical and histologic score were determined. In (G), score (maximal of 9; no inflammation = 2) is defined as the sum of the clinical and histologic score. Clinical score: 1, absence of inflammation; 2, slight rubor and edema; 3, moderate rubor and edema with skin wrinkles; 4, severe rubor and edema without skin wrinkles; and 5, severe rubor and edema with toe spreading. Histologic score (H&E studies): 1, no leukocytic infiltration or subcutaneous edema; 2, slight perivascular leukocytic infiltrate with slight subcutaneous edema; 3, severe leukocytic infiltrate without granulomata; and 4, severe leukocytic infiltrate with granulomata. Mean  $\pm$  SD of  $n = 3$ /group is shown.

albumin was without effect on cell migration (Figure 2F, line 1). EN-RAGE induction of cell migration was due to true chemotaxis, as distortion of the chemotactic gradient by addition of EN-RAGE to both upper and lower compartments suppressed cell movement (Figure 2F, left panel, line 5). The central role of RAGE in EN-RAGE-mediated cell migration was shown by the inhibitory effect observed in the presence of sRAGE (Figure 2F, right panel, lines 2–3) and anti-RAGE F(ab)<sub>2</sub> (Figure 2F, right panel, lines 5 and 6); in both settings, monocyte migration was significantly suppressed compared with incubation with either excess BSA or nonimmune F(ab)<sub>2</sub> (Figure 2F, right panel, lines 1 and 4, respectively). At the site of an ongoing inflammatory response, proinflammatory cytokines, such as IL-1 $\beta$  and TNF $\alpha$ , have been shown to have a central role. Incubation of mock-transfected (vector alone) cultured murine macrophage-like BV2 cells with EN-RAGE caused elaboration of IL-1 $\beta$  into cellular supernatants in a dose-dependent manner (Figure 2G, left panel, filled bars). That intact intracellular signaling pathways triggered by RAGE were essential for IL-1 $\beta$  expression was demonstrated by the suppression observed following transfection of a construct encoding DN-RAGE (Figure 2G, left panel, hatched bars). Similar results were observed when BV-2 cells were analyzed for the effect of EN-RAGE on expression of TNF $\alpha$ ; EN-RAGE-RAGE interaction increased elaboration of TNF $\alpha$

into cellular supernatants in a dose-dependent manner (Figure 2G, right panel, filled bars). However, upon transient transfection with the RAGE-tail deletion construct, BV-2 cell elaboration of TNF $\alpha$  into culture supernatants was significantly suppressed (Figure 2G, right panel, hatched bars). In view of EN-RAGE-mediated induction of cytokine expression via RAGE in BV-2 cells, studies were performed to analyze NF- $\kappa$ B activation under these conditions. As in the studies described above with endothelial cells, EN-RAGE was a strong agonist for induction of nuclear translocation of NF- $\kappa$ B in mononuclear phagocytes; furthermore, blocking access of the ligand to RAGE (with excess sRAGE or anti-RAGE F(ab)<sub>2</sub>) or inhibition of RAGE signaling (by transfection with the construct encoding DN-RAGE) suppressed NF- $\kappa$ B activation (data not shown).

#### PBMCs

The emerging pattern of EN-RAGE-dependent cellular activation, occurring via RAGE, was next explored on PBMCs. PBMCs exposed to EN-RAGE displayed enhanced elaboration of IL-2 into culture supernatant in a RAGE-dependent manner (Figure 2H). Consistent with these findings, an enhanced mitogenic response to cross-linking CD3/CD28 after stimulation of PBMCs with EN-RAGE was noted; compared with pretreatment with albumin (BSA), significant uptake of [<sup>3</sup>H]thymidine was observed in cells preincubated with EN-RAGE, 5 µg/ml

(39,285  $\pm$  2,323 [SD] versus 67,242  $\pm$  1,727 counts per minute [cpm], respectively,  $p < 0.00001$ ). That this was largely due to interaction of EN-RAGE with RAGE expressed by PBMCs was shown by the inhibitory effect of anti-RAGE IgG (30  $\mu$ g/ml) or excess sRAGE (40-fold); suppression of uptake of [ $^3$ H]thymidine was noted (42,413  $\pm$  2,829 and 39,998  $\pm$  3,603 cpm, respectively;  $p < 0.00001$  in both cases). In contrast, treatment with nonimmune F(ab')<sub>2</sub> was without effect (63,642  $\pm$  7,062 cpm;  $p > 0.05$ ).

Taken together, these studies in cell culture model systems demonstrated that engagement of RAGE on the surface of cells critical to pathogenesis of the inflammatory response resulted in acquisition of a phenotype propagating host effector mechanisms, including cell migration, proliferation, and generation of cytokines.

#### Infusion of EN-RAGE Stimulates Cellular Activation

As RAGE bound EN-RAGE and another S100 protein (S100B), resulting in changes in activation of immune/inflammatory effector cells *in vitro*, it was essential to extrapolate our observations to animal models. As a first step in evaluating the *in vivo* relevance of our observations, RAGE-dependent cellular activation was analyzed following intravenous infusion. Systemic administration of EN-RAGE (30  $\mu$ g) to immunocompetent CF-1 mice resulted in an  $\sim 2.4$  increase in expression of VCAM-1 antigen in the lung compared with infusion of albumin (Figure 3A, lanes 2 and 1, respectively). That this was largely due to engagement of RAGE was demonstrated by suppression of EN-RAGE-stimulated VCAM-1 expression in the lung when animals also received sRAGE (Figure 3A, lane 3) or anti-RAGE IgG (Figure 3A, lane 4). Infusion of nonimmune IgG was without effect (Figure 3A, lane 5).

Consistent with *in vitro* findings suggesting that EN-RAGE might modulate inflammatory processes, local injection of EN-RAGE into murine footpad resulted in influx of inflammatory cells (Figure 3C). That these were largely due to interaction with cellular RAGE was evident upon intraperitoneal administration of either sRAGE or anti-EN-RAGE/anti-RAGE F(ab')<sub>2</sub> (Figures 3D, 3F, and 3G, lines 2 and 4, respectively). In contrast, murine serum albumin or nonimmune F(ab')<sub>2</sub> was without effect (Figures 3E and 3G, lines 1 and 3, respectively). These data suggested that EN-RAGE engagement of RAGE occurred *in vivo*. We next tested the pathophysiologic significance of this interaction in models of inflammation.

#### Role of EN-RAGE and RAGE in the Inflammatory Response

##### Acute Inflammation

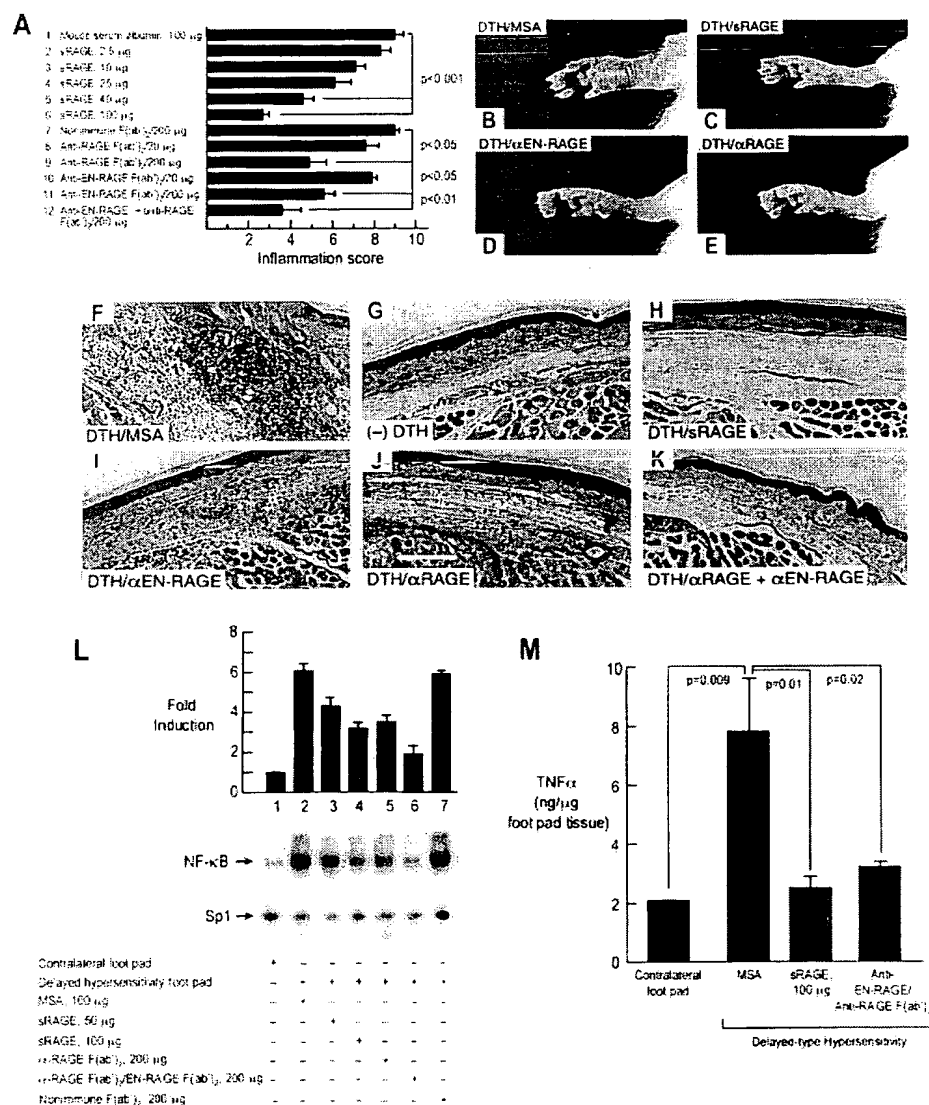
Experimentally induced delayed-type hypersensitivity (Dunn et al., 1993) provided an ideal setting for evaluating the contribution of EN-RAGE interaction with RAGE to an important immune/inflammatory response. CF-1 mice were sensitized with methylated BSA (mBSA; not a ligand of RAGE). Twenty-one days later, mBSA or vehicle (PBS) was injected into the left hind footpad. Mice treated in this manner displayed a strong inflammatory response as measured by the inflammation score

(Figures 4A, 4B, and 4F). Injection of vehicle alone, or administration of mBSA without prior sensitization did not elicit an inflammatory response (data not shown).

The contribution of EN-RAGE interaction with RAGE to the pathogenesis of inflammation in this model was tested by treating mice, prepared by presensitization and challenge with mBSA, with intraperitoneally administered sRAGE or antibodies to RAGE and/or EN-RAGE. Control studies in which the intraperitoneal injection contained vehicle (murine serum albumin [MSA]) demonstrated a strong inflammatory response (score, 9.0  $\pm$  0.4) (Figure 4A, line 1; and Figure 4B) comparable to that observed without an intraperitoneal injection (data not shown). Histologic analysis of the affected footpad demonstrated a striking influx of inflammatory cells, with granulomata and significant edema (Figure 4F). Administration of murine sRAGE by intraperitoneal injection resulted in dose-dependent suppression of inflammation; at an sRAGE concentration of 100  $\mu$ g/dose, the inflammation score was reduced to 2.7  $\pm$  0.3, ( $p < 0.001$  compared with MSA), virtually to background levels (Figure 4A, lines 2–6, and Figure 4C). Consistent with these results, H&E staining revealed striking abrogation of inflammatory cell influx in animals treated with sRAGE (100  $\mu$ g; Figure 4H).

Because it was likely that sRAGE interrupted the EN-RAGE/RAGE axis by binding up EN-RAGE-like species and preventing their interaction with cell surface RAGE, experiments were next performed with specific antibodies to block either EN-RAGE or RAGE. When mice sensitized/challenged with mBSA were treated intraperitoneally with nonimmune F(ab')<sub>2</sub>, no effect was noted on the observed inflammatory response (score, 9.0  $\pm$  0.2; Figure 4A, line 7). However, in the presence of anti-RAGE F(ab')<sub>2</sub> (200  $\mu$ g) or anti-EN-RAGE F(ab')<sub>2</sub> (200  $\mu$ g) significant attenuation of the inflammatory response was evident (Figure 4A, lines 9 and 11, respectively, and Figures 4E and 4D, respectively), with inflammation scores of 4.9  $\pm$  0.8 and 5.6  $\pm$  0.5, respectively ( $p < 0.05$  in both cases compared with treatment with nonimmune F(ab')<sub>2</sub>). Consistent with these results, a striking reduction in the accumulation of inflammatory cells and edema, as well as absence of granulomata, was observed in the presence of either anti-RAGE F(ab')<sub>2</sub> or anti-EN-RAGE F(ab')<sub>2</sub> (Figures 4J and 4I, respectively). In support of a central role for the EN-RAGE/RAGE axis in pathogenesis of the inflammatory response, markedly attenuated inflammation was observed when both anti-EN-RAGE and anti-RAGE F(ab')<sub>2</sub> were administered simultaneously (Figure 4A, line 12); inflammation score was reduced to 3.6  $\pm$  0.9 ( $p < 0.01$ , compared with treatment with nonimmune F(ab')<sub>2</sub>), with decreased numbers of inflammatory cells and edema (Figure 4K).

These data suggested that blockade of EN-RAGE interaction with RAGE substantially quenched cellular activation in a model of delayed-type hypersensitivity. In parallel with suppressed inflammation in mBSA-sensitized/challenged mice treated with either sRAGE, anti-RAGE F(ab')<sub>2</sub>, or anti-RAGE/anti-EN-RAGE F(ab')<sub>2</sub>, suppression of NF- $\kappa$ B activation was observed in nuclear extracts prepared from the affected footpads. Compared with the contralateral footpad (the latter prepared by sensitization with mBSA, but without local challenge),



**Figure 4. Blockade of EN-RAGE/RAGE Suppresses Inflammation in a Model of Delayed-Type Hypersensitivity**

(A) Inflammation score. CF-1 mice were sensitized and challenged with mBSA. Mice were pretreated by intraperitoneal injection with sRAGE, MSA, immune or nonimmune F(ab')<sub>2</sub> fragments prior to and after local challenge with mBSA. Twenty-four hours after injection of footpad with mBSA, clinical and histologic score of footpad were performed employing the criteria in Figure 3.

(B-E) Representative mice sensitized/challenged with mBSA are shown: (B), treatment with MSA; (C), treatment with sRAGE, 100 μg IP per dose; (D), treatment with anti-EN-RAGE F(ab')<sub>2</sub>, 200 μg IP per dose; and (E), treatment with anti-RAGE F(ab')<sub>2</sub>, 200 μg IP per dose.

(F-K) H&E analysis of representative footpads from mice sensitized/challenged with mBSA is shown: (F), treatment with MSA; (G), contralateral footpad, no DTH; (H), treatment with sRAGE, 100 μg IP per dose; (I), anti-EN-RAGE F(ab')<sub>2</sub>, 200 μg IP per dose; (J), anti-RAGE F(ab')<sub>2</sub>, 200 μg IP per dose; and (K), anti-EN-RAGE + anti-RAGE F(ab')<sub>2</sub>, 200 μg IP per dose. Bar, 150 μm.

(L) EMSA. Nuclear extracts were prepared from pooled hind footpads (n = 3/condition), and EMSA was performed for NF-κB normalized to Sp1. Mean ± SD of three experiments is shown.

(M) Measurement of TNFα. Extract of indicated footpad was prepared, and ELISA for murine TNFα was performed. Mean ± SD is reported.

nuclear extracts from the mBSA-injected footpad revealed an ~6-fold increase in activation of NF-κB (Figure 4L, lanes 1 and 2, respectively). In the presence of sRAGE (100 μg/dose; IP), the intensity of the gel shift band was substantially reduced as contrasted with results using nuclear extracts from footpads appropriately

prepared/challenged with mBSA but treated only with vehicle (MSA; Figure 4L, lanes 4 and 2, respectively). Consistent with the protective effect of interrupting EN-RAGE interaction with RAGE, administration of anti-RAGE/anti-EN-RAGE F(ab')<sub>2</sub> to mBSA-sensitized/challenged mice caused an ~70% decrease in activation of



Table 2. Histologic Examination of Rectosigmoid Tissue Retrieved from IL-10 Null Mice

Mouse No.	Condition	Cryptitis	Cellular Infiltrate	Comments
1	MSA	Absent	M	Two clusters of mononuclear cells identified
2	MSA	Present (base)	L,P,E,M	Patchy submucosal inflammation
3	MSA	Absent	L,P,E,M	Patchy submucosal inflammation
4	MSA	Present (base)	L,P,E,M	Focal submucosal inflammation
5	MSA	Absent	None	No inflammation
6	sRAGE	Absent	None	No inflammation
7	sRAGE	Present (base)	L,P,E,M	Patchy submucosal inflammation
8	sRAGE	Absent	None	No inflammation
9	sRAGE	Absent	None	No inflammation
10	sRAGE	Absent	None	No inflammation

Rectosigmoid tissue was retrieved from IL-10 null mice treated with either murine serum albumin (MSA) or sRAGE as indicated. Sections were prepared as described and stained with H&E; histologic scoring was performed by a blinded investigator. Inflammatory cells are identified as follows: M, monocyte/macrophage; L, lymphocyte; E, eosinophil; and P, plasma cell.

NF- $\kappa$ B (Figure 4L, lane 6) versus that seen in the presence of nonimmune F(ab')<sub>2</sub> (Figure 4L, lane 7). These data indicate that blocking binding of EN-RAGE to RAGE potentially quenched signaling via the NF- $\kappa$ B pathway.

An important consequence of RAGE ligation by EN-RAGE was increased expression of inflammatory mediators, likely due, at least in part, to NF- $\kappa$ B. Thus, expression of the proinflammatory cytokine TNF $\alpha$  was compared in footpad protein extracted from mBSA-sensitized/challenged mice. Compared with mice receiving vehicle (MSA), animals treated with sRAGE (100  $\mu$ g) displayed an  $\sim$ 3.1-fold decrease in levels of TNF $\alpha$  protein in footpad tissue (Figure 4M). Similar beneficial effects were observed upon treatment with anti-EN-RAGE/anti-RAGE F(ab')<sub>2</sub> (Figure 4M).

#### Chronic Inflammation

A hallmark of S100/calgranulin polypeptides is their association with chronic inflammation, such as human inflammatory bowel diseases (Lugering et al., 1995). To determine whether S100/calgranulins were contributing to pathogenesis of the inflammatory lesions via their interaction with RAGE, a murine model of colitis, IL-10 null mice (Kuehn et al., 1993), was employed and RAGE blockade was achieved by administration of sRAGE. IL-10 null mice were treated with either MSA or sRAGE (100  $\mu$ g per day; IP) for 6 weeks, and, at the end of that time, rectosigmoid colon was assessed for evidence of inflammation. Although 4/5 mice receiving MSA displayed submucosal colonic inflammatory infiltrates composed of lymphocytes, macrophages, eosinophils, and plasma cells, 4/5 mice treated with sRAGE showed virtually no inflammation (Table 2). Consistent with these histologic results, nuclear extracts prepared from colonic tissue of IL-10 null treated with MSA and subjected to EMSA with the <sup>32</sup>P-NF- $\kappa$ B probe displayed a strong gel shift band in 5/6 mice (Figure 5A, lanes 7–12). Following treatment with sRAGE, virtually complete suppression of the gel shift band was observed in 3/6 animals (Figure 5A, lanes 3–4 and 6), and, taking all animals into account (Figure 5A, lanes 1–12), the presence of sRAGE reduced the intensity of the bands by  $\sim$ 75% ( $p = 0.04$ ). Paralleling these results, an  $\sim$ 8.7-fold decrease in plasma levels of TNF $\alpha$  was observed in mice treated with sRAGE compared with those receiving MSA ( $p = 0.002$ ; Figure 5B).

#### Discussion

The presence of S100/calgranulin polypeptides at sites of acute and chronic inflammation has long been noted.

Indeed, assessment of serum levels of MRP8/14 (myeloid-related protein), S100-like molecules, has been suggested to track disease activity in patients with ulcerative colitis, a chronic inflammatory disease of the bowel (Lugering et al., 1995). S100/calgranulin molecules have in common structural features, most characteristic of which are calcium-binding EF-hand domains. Based on these properties, a range of possible intracellular functions for these polypeptides has been postulated, such as alteration of the cytoskeleton and cell shape, signal transduction, and, via modulation of intracellular calcium, regulation of chemotaxis, phagocytosis, and generation of reactive oxygen species (ROIs) (Schafer and Heinzmann, 1996).

Despite the fact that EN-RAGE and related family

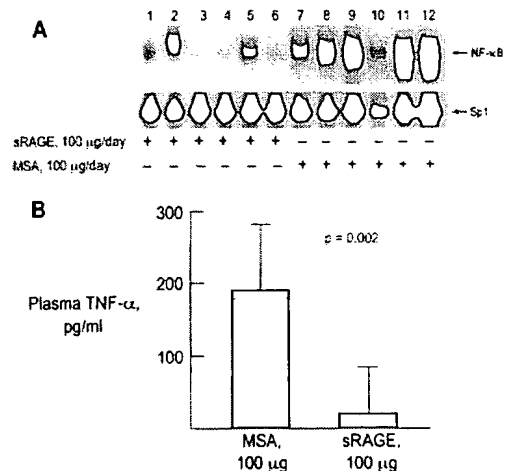


Figure 5. Blockade of EN-RAGE/RAGE Suppresses Chronic Colonic Inflammation in IL-10 Null Mice

(A) EMSA. Nuclear extracts were prepared from rectosigmoid colon tissue of mice treated with either sRAGE (lanes 1–6) or MSA (lanes 7–12). Densitometric analysis after normalization for Sp1 was performed. Mean densitometry pixel units for MSA-treated ( $n = 6$ ) versus sRAGE-treated mice ( $n = 6$ ) were 7121.8  $\pm$  5359.6 versus 1,911  $\pm$  1155 U;  $p = 0.04$ .

(B) Assessment of plasma TNF $\alpha$ . Plasma was retrieved from IL-10 null mice upon sacrifice, and ELISA for TNF $\alpha$  was performed. Mean values for MSA-treated ( $n = 6$ ) versus sRAGE-treated mice ( $n = 6$ ) were 190.5  $\pm$  89.0 versus 21.9  $\pm$  63.6 pg/ml, respectively;  $p = 0.002$ .

members lack signal peptides, there is definitive evidence that these polypeptides readily gain access to the extracellular space (Rammes et al., 1997) and mediate an array of inflammatory phenomena. For example, following infusion or injection of CP-10 (chemotactic peptide-10; a member of the S100 family) into mice, analysis of elicited peritoneal macrophages revealed increased expression of the scavenger receptor and TNF $\alpha$ , enhanced loading of acetylated LDL and foam cell formation, increased phagocytosis, and local footpad inflammation (Geczy, 1996). The data presented here suggest a novel pathway through which S100/calgranulin molecules released into the extracellular space contribute to the pathogenesis of the inflammatory response via interaction with RAGE-bearing cells. The binding of EN-RAGE and other EN-RAGE-like molecules (S100/calgranulin family members) to RAGE on critical cellular targets activates signaling pathways, thereby modulating gene expression and amplifying inflammatory effector mechanisms. Since both S100/calgranulins and RAGE are associated with chronic inflammation, this suggests possible roles for EN-RAGEs, via engagement of RAGE, in sustaining the inflammatory response in a range of disorders beyond traditional inflammatory conditions, such as inflammatory bowel disease, to atherosclerosis (Ross, 1999), Alzheimer's disease (Marshak et al., 1992), and other situations in which an inflammatory component has gained increasing recognition. Although it is not yet clear whether RAGE will bind all S100/calgranulins, as EN-RAGE most closely resembles calgranulins and S100B is an S100 protein, it is likely that the receptor will interact with other family members. Indeed, our studies indicate that anti-EN-RAGE IgG recognizes both bovine and human S100B in immunoblotting studies (data not shown). Further, although our data do not rule out a role for RAGE-independent mechanisms of S100/calgranulin-mediated cellular perturbation, the inhibitory effect of anti-RAGE F(ab')<sub>2</sub> and sRAGE on the inflammatory response emphasizes an important role for RAGE.

The present findings expand the biologic contexts of RAGE as a cell surface immunoglobulin superfamily member with a distinct panel of ligands whose functional implications extend to both development and pathophysiologically relevant states (Schmidt et al., 1998). Indeed, assignment of RAGE to the major histocompatibility complex on chromosome 6 (Sugaya et al., 1994) suggested a possible relationship of this receptor to immune/inflammatory responses. The presence of functional NF- $\kappa$ B sites in the RAGE promoter, as well as putative NF-IL-6,  $\gamma$ -IRE, and AP2-binding sites further reinforced the likelihood that RAGE would contribute to the pathogenesis of inflammation (Li and Schmidt, 1997; Li et al., 1998). Our present findings close this loop by demonstrating that RAGE engagement of S100/calgranulins (EN-RAGEs) at inflammatory loci propagates the host response by perpetuating recruitment and activation of cellular effectors. By placing RAGE at the center of chronic inflammation, a novel paradigm can be proposed and tested concerning cellular effects of S100/calgranulins and their contribution to the pathogenesis of diverse disorders, from inflammatory bowel disease and delayed-type hypersensitivity, to Alzheimer's disease, atherosclerosis, and the complications of diabetes.

## Experimental Procedures

### Protein Sequence Analysis

To perform sequence analysis of RAGE-binding proteins from bovine lung extract, Coomassie blue visible bands (~12 kDa) were eluted from SDS-PAGE gels. Automated Edman degradation was carried out using an HP-G1005A sequencer (Hewlett Packard Analytical Instruments, Palo Alto, CA). Internal sequencing was performed employing endoproteinase Lys-C (Boehringer Mannheim, Indianapolis, IN) digestion followed by microbore HPLC (Hori et al., 1995).

### Molecular Cloning

Molecular cloning was performed using bovine and human lung libraries (Clontech, Palo Alto, CA) in order to obtain cDNAs for EN-RAGE. The sequence encoding bovine cDNA for EN-RAGE is No. AF011757 (GenBank).

### Protein Expression

The cDNA encoding bovine EN-RAGE was placed into a baculovirus expression system and expressed in *Spodoptera frugiperda* 9 (Sf9) cells (Invitrogen, Carlsbad, CA). EN-RAGE was purified from cellular pellets by sequential chromatography onto heparin and hydroxylapatite columns (Amersham Pharmacia, Piscataway, NJ) and eluted with increasing concentrations of NaCl. Purified EN-RAGE, a single band on Coomassie-stained gels ( $M_r$  = 12 kDa) was devoid of endotoxin prior to experiments by chromatography onto Detox-gel columns (Pierce, Arlington Heights, IL) as documented using a kit from Sigma (St. Louis, MO) (limulus amebocyte assay). Purified S100B from human brain was obtained from Calbiochem-Novabiochem Corp. (San Diego, CA). Recombinant murine soluble RAGE was prepared as described (Park et al., 1998).

### Immunoblotting

#### In Vitro Studies

Human peripheral blood-derived mononuclear cells were isolated from normal volunteers by using Histopaque 1077 (Sigma), and Jurkat E6 cells were obtained from the American Type Tissue Corporation (Rockville, MD). Where indicated, PBMCs or Jurkat E6 cells were stimulated by cross-linking CD3/CD28 (Gimmi et al., 1991). Briefly, cells were incubated with purified mouse anti-human CD3 IgG (1  $\mu$ g/ml) (PharMingen, San Diego, CA) for 24 hr followed by washing in PBS, and then incubated with 1  $\mu$ g/ml purified mouse anti-human CD28 IgG (PharMingen) for an additional 24 hr. Supernatant was retrieved and assayed for levels of IL-2 by ELISA (R&D Systems, Minneapolis, MN). HUVECs were stimulated with TNF $\alpha$  (10 ng/ml) (Genzyme, Cambridge, MA) for 12 hr. In all cases, after stimulation, cells ( $1 \times 10^7$ ) were sonicated (Sonifer 250, Branson, Danbury, CT) in PBS containing protease inhibitor mixture (Boehringer Mannheim) and centrifuged for 30 min at 14,000 rpm at 4°C. Protein concentration was measured using Bio-Rad protein assay (Hercules, CA). To each lane of Tris-glycine gels (Novex, San Diego, CA), 7  $\mu$ g of protein was added; gel components were transferred to nitrocellulose membranes (Bio-Rad) and immunoblotting performed using polyclonal rabbit monospecific anti-EN-RAGE IgG prepared against full-length recombinant bovine EN-RAGE. Goat anti-rabbit IgG labeled with horseradish peroxidase (Sigma) and ECL system (Amersham-Pharmacia) were employed to indicate sites of primary antibody binding.

#### In Vivo Studies

LPS (Sigma) (30  $\mu$ g/g body weight) was injected intraperitoneally into CF-1 mice. Mice were sacrificed at the indicated time points and serum (0.015 ml) subjected to electrophoresis using Tris-glycine gels (14%; Novex) and immunoblotting for detection of EN-RAGE performed as above. Densitometry was performed using ImageQuant, Molecular Dynamics (Foster City, CA).

### Radioligand Binding Assays

Purified EN-RAGE was radiolabeled using <sup>125</sup>I and Iodobeads (Pierce) to a specific activity of ~5000 cpm/ng. Radioligand binding assays were performed in 96-well dishes to which had been adsorbed purified murine recombinant soluble RAGE (5  $\mu$ g/well). Radioligand binding assays were performed in the presence of the

indicated concentration of radiolabeled EN-RAGE  $\pm$  a 50-fold molar excess of unlabeled EN-RAGE, amphotericin, amyloid- $\beta$ -peptide 1–40, or AGE albumin in PBS containing calcium/magnesium and BSA (0.2%) for 3 hr at 37°C. Wells were washed rapidly, and elution of bound material was performed in the presence of heparin (1 mg/ml). Eluate was counted in a gamma counter (LKB, Gaithersburg, MD). Equilibrium binding data were analyzed as described (Klotz and Hunston, 1984):  $B = nKA / (1 + KA)$ , where  $B$  = specifically bound ligand (total binding, wells incubated with tracer alone, minus non-specific binding, wells incubated with tracer in the presence of excess unlabeled material),  $n$  = sites/cell,  $K$  = the dissociation constant, and  $A$  = free ligand concentration) using nonlinear least-squares analysis (Prism; San Diego, CA). Where indicated, pretreatment with either antibodies or soluble RAGE (2 hr) was performed.

#### Cellular Activation Studies

##### Endothelial Cells

HUVECs were isolated and characterized as described (Schmidt et al., 1995) and cultured in serum-free RPMI 1640 without endothelial cell growth factor for 24 hr and then stimulated with the indicated concentrations of EN-RAGE or other stimuli. Certain cells were pretreated with rabbit anti-human RAGE IgG or nonimmune rabbit IgG for 2 hr, or EN-RAGE was pretreated with sRAGE for 2 hr prior to stimulation with EN-RAGE. After 8 hr, cells were fixed with paraformaldehyde (2%) for 30 min, washed twice with PBS, and treated with PBS containing nonfat dry milk (5%) and BSA (2.5%) to block nonspecific binding sites on the cell surface. Cell surface ELISA employing anti-VCAM-1 IgG (4  $\mu$ g/ml; Santa Cruz Biotechnologies, Santa Cruz, CA) was performed and VCAM-1 activity determined using  $^{125}$ I-labeled Molt-4 cells (ATCC) (Schmidt et al., 1995). Levels of ICAM-1 in EN-RAGE-treated HUVECs were determined by immunoblotting with anti-ICAM-1 IgG (Santa Cruz; 0.2  $\mu$ g/ml) after 12 hr incubation. EMSA was performed with 10  $\mu$ g nuclear extract loaded onto PAGE gels in the presence of  $^{32}$ P-labeled probe for NF- $\kappa$ B from the VCAM-1 promoter (Neish et al., 1992) or radiolabeled probe for Sp1 (Santa Cruz). Supershift assays were performed by preincubating nonimmune anti-p50, anti-p65, or both IgG (2  $\mu$ g/ml) (Santa Cruz) with nuclear extract for 45 min prior to addition of radiolabeled oligonucleotide probe.

##### PBMCs and MPs

**Chemotaxis Assays.** Chemotaxis assays were performed in 48-well microchemotaxis chambers (Neuro-Probe, Bethesda, MD) containing a polycarbonate membrane (8  $\mu$ m; Nucleopore, Pleasanton, CA) (Miyata et al., 1996). The lower chamber contained the chemotactic stimulus. N-formyl-met-leu-phe (Sigma) was employed as positive control. Human MPs were added to the upper chamber (10<sup>4</sup> cells/well). After 4 hr at 37°C, nonmigrating cells on the upper surface of the membrane were gently scraped and removed, the membrane was fixed in methanol (100%), and cells that had migrated through the membrane were stained with Giemsa (Sigma) and counted in nine high-powered fields.

**Mitogenic Assays.** PBMCs were isolated from whole blood and suspended in RPMI containing FBS (10%) (1  $\times$  10<sup>6</sup> cells/ml). Cells were seeded in 96-well tissue culture wells and treated with EN-RAGE for 12 hr prior to stimulation by cross-linking CD3/CD28 as above for 12 hr. Wells were pulsed with [<sup>3</sup>H]thymidine (1  $\mu$ Ci/well) (New England Nuclear, Boston, MA) and incubated for an additional 24 hr prior to harvesting and processing for liquid scintillation counting using an LK betaplate (Wallac, Inc., Gaithersburg, MD).

**Assessment of Cytokines.** RAGE-bearing BV2 cells (Yan et al., 1997) or PBMCs were incubated with the indicated mediators; certain cells were preincubated with anti-RAGE F(ab')<sub>2</sub> prior to stimulation with EN-RAGE. In other cases, EN-RAGE was preincubated with sRAGE prior to stimulation. ELISA for TNF $\alpha$ , IL-1 $\beta$ , or IL-2 was performed (R&D Systems). Where indicated, cells were transfected with a construct encoding human RAGE in which the cytosolic domain (tail) was deleted employing superfect (Qiagen, Valencia, CA) (1  $\mu$ g DNA/ml); pcDNA3 (Invitrogen) was employed as vector. Stimulation experiments were performed 48 hr after transfection.

#### Infusion Studies and Local EN-RAGE-Mediated Inflammation

Male CF-1 mice (Charles River), approximately 6 weeks of age, were injected via the tail vein with EN-RAGE (30  $\mu$ g), BSA (30  $\mu$ g), or LPS

(500  $\mu$ g). Twelve hours later, lungs were harvested, and extract was subjected to electrophoresis/immunoblotting employing anti-VCAM-1 IgG (Santa Cruz). In other studies, EN-RAGE (10  $\mu$ g) or vehicle PBS was injected into hind footpad. Certain animals were pretreated with sRAGE, MSA, or the indicated F(ab')<sub>2</sub> fragments 12 hr prior to injection, at the time of injection, and 12 hr after injection. Mice were sacrificed 24 hr after injection; clinical and histologic scores of H&E-fixed tissue were performed by two blinded investigators.

#### Model of Delayed Hypersensitivity

Female CF-1 mice, 6 weeks of age, were sensitized by subcutaneous injection over the left inguinal lymph node of an emulsion (0.1 ml) containing methylated BSA (mBSA; 25 mg/ml; Sigma), NaCl (0.9%), dextran (5–40  $\times$  10<sup>6</sup> MW; 50 mg/ml; Sigma), and Freund's incomplete adjuvant (50%; ICN Biomedical; Aurora, OH). Three weeks later, the left plantar hind paw was injected subcutaneously with mBSA (0.4 mg/ml; 0.050 ml). Where indicated, mice were pretreated by intraperitoneal injection with sRAGE, mouse serum albumin, immune or nonimmune F(ab')<sub>2</sub> fragments, 24 and 12 hr prior to, and 6 and 12 hr after local challenge with mBSA. Twenty-four hours after injection of footpad with mBSA, clinical score of footpad was performed by two blinded investigators; mice were humanely sacrificed and feet fixed in formalin (10%) or frozen. Histologic score was performed on sections of footpad stained with hematoxylin and eosin (Sigma) by two blinded investigators. Electrophoretic mobility shift assay was performed employing 10  $\mu$ g footpad nuclear extract added per lane and normalized to Sp1. Assessment of tissue extract prepared as above for levels of TNF $\alpha$  was performed.

#### Model of Chronic Colitis in IL-10 Null Mice

Four-week-old IL-10 null mice (C57BL/6 background) (Jackson Laboratories, Bar Harbor, ME) were treated once daily by intraperitoneal injection for 6 weeks with either MSA (100  $\mu$ g/day) or sRAGE (100  $\mu$ g/day). Mice were deeply anesthetized; plasma was removed, and rectosigmoid colon was retrieved for histologic analysis or preparation of nuclear extracts. Hematoxylin and eosin-stained sections of rectosigmoid were evaluated by a blinded investigator. Plasma was assessed for levels of TNF $\alpha$  (R&D Systems), and EMSA for NF- $\kappa$ B was performed on nuclear extracts.

#### Statistical Analysis

Statistical comparisons among groups was determined using one-way analysis of variance (ANOVA); where indicated, individual comparisons were performed using Student's *t* test. Statistical significance was ascribed to the data when *p* < 0.05.

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#### References

- Brownlee, M., Cerami, A., and Vlassara, H. (1988). Advanced glycosylation endproducts in tissue and the biochemical basis of diabetic complications. *N. Engl. J. Med.* 318, 1315–1320.
- Dell'Angelica, E. C., Schleicher, C. H., and Santome, J. A. (1994). Primary structure and binding properties of calgranulin C, a novel S100-like calcium-binding protein from pig granulocytes. *J. Biol. Chem.* 269, 28929–28936.
- Dunn, C. J., Galinet, L. A., Wu, H., Nugent, R. A., Schlachter, S. T., Staitte, N. D., Aspar, D. G., Elliott, G. A., Essani, N. A., Rohloff, N. A., and Smith, R. J. (1993). Demonstration of novel anti-arthritis and

- anti-inflammatory effects of Diphosphonates. *J. Pharmacol. Exp. Ther.* 266, 1691-1698.
- Geczy, C. (1996). Regulation and proinflammatory properties of chemotactic protein, CP-10. *Biochim. Biophys. Acta* 1313, 246-252.
- Gimmi, C.D., Freeman, G.J., Gribben, J.G., Sugita, K., Freedman, A.S., Morimoto, C., Nadler, L.M. (1991). B-cell surface antigen B7 provides a costimulatory signal that induces T cells to proliferate and secrete interleukin-2. *Proc. Natl. Acad. Sci. USA* 88, 6575-6579.
- Gottsch, J.D., Stark, W.J., and Liu, S.H. (1997). Cloning and sequence analysis of human and bovine corneal antigen (CO-Ag) cDNA: identification of host-parasite protein calgranulin C. *Trans. Am. Ophthalmol. Soc.* 74, 111-129.
- Hitomi, H., Yamaguchi, K., Kikuchi, Y., Kimura, T., Maruyama, K., and Nagasaki, K. (1996). A novel calcium-binding protein in amniotic fluid, CAAF1: its molecular cloning and tissue distribution. *J. Cell Sci.* 109, 805-815.
- Hori, O., Brett, J., Slattery, T., Cao, R., Zhang, J., Chen, J., Nagashima, M., Nitecki, D., Morser, J., Stern, D., and Schmidt, A.M. (1995). The receptor for advanced glycation endproducts (RAGE) is a cellular binding site for amphotericin: mediation of neurite outgrowth and coexpression of RAGE and amphotericin in the developing nervous system. *J. Biol. Chem.* 270, 25752-25761.
- Ilg, E.C., Troxler, H., Buerigisser, D.M., Kuster, T., Markert, M., Guignard, F., Hunziker, P., Birchler, N., and Heinzmann, C.W. (1996). Amino acid sequence determination of human S100A12 (p6, Calgranulin C, CGRP, CAAF1) by tandem mass spectrometry. *Biochem. Biophys. Res. Comm.* 225, 146-150.
- King, G., and Brownlee, M. (1996). The cellular and molecular mechanisms of diabetic complications. *Endocrin. Metab. Clin. North Am.* 25, 255-270.
- Klotz, I., and Hunston, D. (1984). Mathematical models for ligand-receptor binding. *J. Biol. Chem.* 259, 10060-10062.
- Krieger, M., and Herz, J. (1994). Structure and function of multiligand lipoprotein receptors: macrophage scavengers and LDL receptor-related protein. *Annu. Rev. Biochem.* 63, 601-637.
- Kuehn, R., Loehler, J., Rennick, D., Rajewsky, K., and Mueller, W. (1993). Interleukin-10-deficient mice develop chronic enterocolitis. *Cell* 75, 263-274.
- Lander, H.L., Tauras, J.M., Ogiste, J.S., Moss, R.A., and A.M. Schmidt. (1997). Activation of the receptor for advanced glycation endproducts triggers a MAP kinase pathway regulated by oxidant stress. *J. Biol. Chem.* 272, 17810-17814.
- Li, J., and Schmidt, A.M. (1997). Characterization and functional analysis of the promoter of RAGE, the receptor for advanced glycation endproducts. *J. Biol. Chem.* 271, 16498-16506.
- Li, H., Cybulsky, M., Gimbrone, M., and Libby, P. (1993). An atherogenic diet rapidly induces VCAM-1, a cytokine-regulatable mononuclear leukocyte adhesion molecule in rabbit aortic endothelium. *Arterioscler. Thromb.* 13, 197-204.
- Li, J., Qu, X., and Schmidt, A.M. (1998). Sp1 binding elements in the promoter of RAGE are essential for amphotericin-mediated gene expression in cultured neuroblastoma cells. *J. Biol. Chem.* 273, 30870-30878.
- Lugering, N., Stoll, R., Schmid, K.W., Kucharzik, T., Stein, H., Burgmeister, G., Sorg, C., and Domschke, W. (1995). The myeloid related protein MRP8/14 (27E10 antigen) usefulness as a potential marker for disease activity in ulcerative colitis and putative biological function. *Eur. J. Clin. Invest.* 25, 659-664.
- Mackic, J.B., Stins, M., McComb, J.G., Calero, M., Ghiso, J., Kim, K.S., Yan, S.D., Stern, D., Schmidt, A.M., Frangione, B., and Zlokovic, B.V. (1998). Human blood-brain barrier receptors for Alzheimer's amyloid- $\beta$ 1-40: asymmetrical binding, endocytosis, and transcytosis at the apical side of brain microvascular endothelial cell monolayer. *J. Clin. Invest.* 102, 734-743.
- Marshak, D.R., Pesce, S.A., Stanley, L.C., and Griffin, W.S. (1992). Increased S100 beta neurotrophic activity in Alzheimer's disease temporal lobe. *Neurobiol. Aging* 13, 1-7.
- Miyata, T., O'Hori, J.H., Zhang, S.D., Yan, L., Ferran, Y., Iida, and Schmidt, A.M. (1996). The receptor for advanced glycation endproducts (RAGE) mediates the interaction of AGE- $\beta$ 2-microglobulin with human mononuclear phagocytes via an oxidant-sensitive pathway: implications for the pathogenesis of dialysis-related amyloidosis. *J. Clin. Invest.* 98, 1088-1094.
- Neeper, M., Schmidt, A.M., Brett, J., Yan, S.D., Wang, F., Pan, Y.C., Elliston, K., Stern, D., and Shaw, A. (1992). Cloning and expression of RAGE: a cell surface receptor for advanced glycosylation end products of proteins. *J. Biol. Chem.* 267, 14998-15004.
- Neish, A.S., Williams, A.J., Palmer, H.J., Whitley, M.Z., and Collins, T. (1992). Functional analysis of the human vascular cell adhesion molecule-1 promoter. *J. Exp. Med.* 176, 1583-1593.
- Park, L., Raman, K.G., Lee, K.J., Yan, L., Ferran, L.J., Chow, W.S., Stern, D., and Schmidt, A.M. (1998). Suppression of accelerated diabetic atherosclerosis by soluble receptor for AGE (sRAGE). *Nat. Med.* 4, 1025-1031.
- Rammes, A., Roth, J., Goebeler, M., Klempt, M., Hartmann, M., and Sorg, C. (1997). Myeloid-related protein (MRP) 8 and MRP14, calcium-binding proteins of the S100 family, are secreted by activated monocytes via a novel, tubulin-dependent pathway. *J. Biol. Chem.* 272, 9496-9502.
- Rauvala, H., and Pihlaskari, R. (1987). Isolation and some characteristics of an adhesive factor of brain that enhances neurite outgrowth in central neurons. *J. Biol. Chem.* 262, 16625-16635.
- Ross, R. (1999). Atherosclerosis—an inflammatory disease. *N. Engl. J. Med.* 340, 115-126.
- Schafer, B.W., and Heinzmann, C.W. (1996). The S100 family of EF-hand calcium-binding proteins: functions and pathology. *TIBS* 21, 134-140.
- Schmidt, A.M., Vianna, M., Gerlach, M., Brett, J., Ryan, J., Kao, J., Esposito, C., Hegarty, H., Hurley, W., Clauss, M., et al. (1992). Isolation and characterization of binding proteins for advanced glycosylation endproducts from lung tissue which are present on the endothelial cell surface. *J. Biol. Chem.* 267, 14987-14997.
- Schmidt, A.-M., Hori, O., Chen, J., Li, J., Crandall, J., Zhang, J., Cao, R., Yan, S.D., Brett, J., and Stern, D. (1995). Advanced glycation endproducts interacting with their endothelial receptor induce expression of vascular cell adhesion molecule-1 (VCAM-1): a potential mechanism for the accelerated vasculopathy of diabetes. *J. Clin. Invest.* 96, 1395-1403.
- Schmidt, A.M., Wautier, J.-L., Stern, D., and Yan, S.D. (1998). RAGE: a receptor with a taste for multiple ligands and varied pathophysiological states. *Horm. Signal.* 1, 41-63.
- Selkoe, D. (1994). Alzheimer's disease: a central role for amyloid. *J. Neuropathol. Exp. Neurol.* 53, 438-447.
- Springer, T. (1990). Adhesion receptors of the immune system. *Nature* 346, 425-434.
- Sugaya, K., Fukagawa, T., Matsumoto, K., Mita, K., Takahashi, E., Ando, A., Inoko, H., and Ikemura, T. (1994). Three genes in the human MHC class III region near the junction with the class II: gene for receptor for advanced glycosylation end products, PBX2 homeobox 2 gene and a notch homolog, human counterpart of mouse mammary tumor gene int-3. *Genomics* 23, 408-419.
- Voraberger, G., Schafer, R., and Stratowa, C. (1991). Cloning of the human gene for intercellular adhesion molecule 1 and analysis of the 5'-regulatory region. Induction by cytokines and phorbol ester. *J. Immunol.* 147, 2777-2786.
- Wautier, J.-L., Zoukourian, C., Chappey, O., Wautier, M.P., Guillausseau, P.J., Cao, R., Hori, O., Stern, D., and Schmidt, A.M. (1996). Receptor-mediated endothelial cell dysfunction in diabetic vasculopathy: soluble receptor for advanced glycation endproducts blocks hyperpermeability. *J. Clin. Invest.* 97, 238-243.
- Wicki, R., Marenholz, I., Mischke, D., Schaefer, B.W., Heinzmann, C.W. (1996). Characterization of the human S100A12 (calgranulin C, p6, CAAF1, CGRP) gene, a new member of the S100 gene cluster on chromosome 1q21. *Cell Calcium* 20, 459-464.
- Yamamura, T., Hitomi, J., Nagasaki, K., Suzuki, M., Takahashi, E., Saito, S., Tsukada, T., and Yamaguchi, K. (1996). Human CAAF1 gene-molecular cloning, gene structure, and chromosome mapping. *Biochem. Biophys. Res. Comm.* 221, 356-360.
- Yan, S.D., Schmidt, A.-M., Anderson, G., Zhang, J., Brett, J., Zou, Y.-S., Pinsky, D., and Stern, D. (1994). Enhanced cellular oxidant

stress by the interaction of advanced glycation endproducts with their receptors/binding proteins. *J. Biol. Chem.* 269, 9889–9897.

Yan, S.D., Chen, X., Fu, J., Chen, M., Zhu, H., Roher, A., Slattery, T., Nagashima, M., Morser, J., Migheli, A., et al. (1996). RAGE and amyloid beta peptide neurotoxicity in Alzheimer's disease. *Nature* 382, 685–691.

Yan, S.D., Zhu, H., Fu, J., Yan S.F., Roher, A., Tourtellotte, W., Rajavashisth, T., Chen, X., Stern, D., and Schmidt, A.M. (1997). Amyloid-beta peptide-RAGE interaction elicits neuronal expression of M-CSF: a proinflammatory pathway in Alzheimer's disease. *Proc. Natl. Acad. Sci. USA* 94, 5296–5301.

Zimmer, D.B., Cornwall, E.H., Landar, A., and Song, W. (1995). The S100 protein family: history, function, and expression. *Brain Res. Bull.* 37, 417–429.